

D. Williams, et al.
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IN THE DRAWINGS:

Replacement sheets are enclosed for Figures 1, 3, 4, 8, 9, 17, 21, 22, 24, 28, 31, 32, 33, 34, 35, 36 and 37.

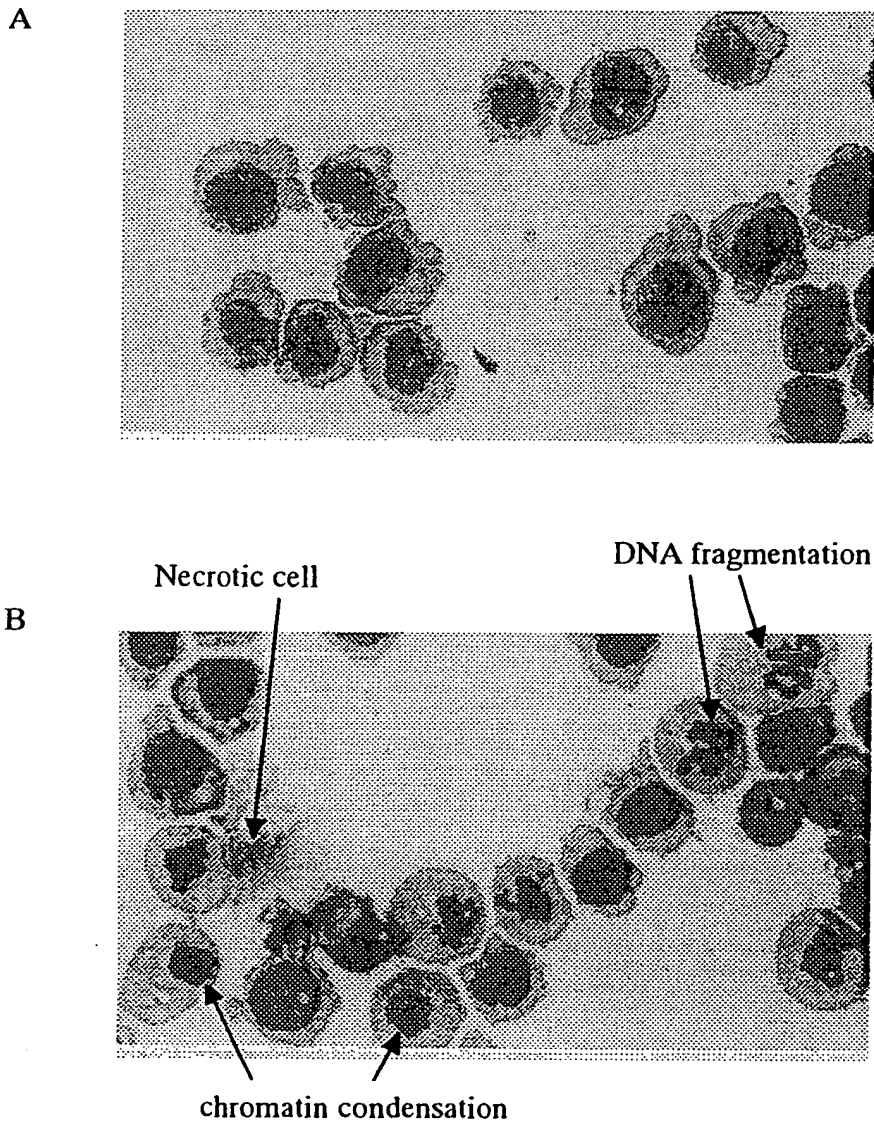


Fig. 1 Morphological features of HL-60 cells undergoing apoptosis following treatment with PBOX-6.

Microscopic analysis of HL-60 cells was performed on cytospin samples. Vehicle (1% ethanol) treated cells (A) are characterised by a continuous plasma membrane and an intact nucleus. PBOX-6 treated cells (B) display the morphological features of apoptosis, which includes chromatin condensation and DNA fragmentation.

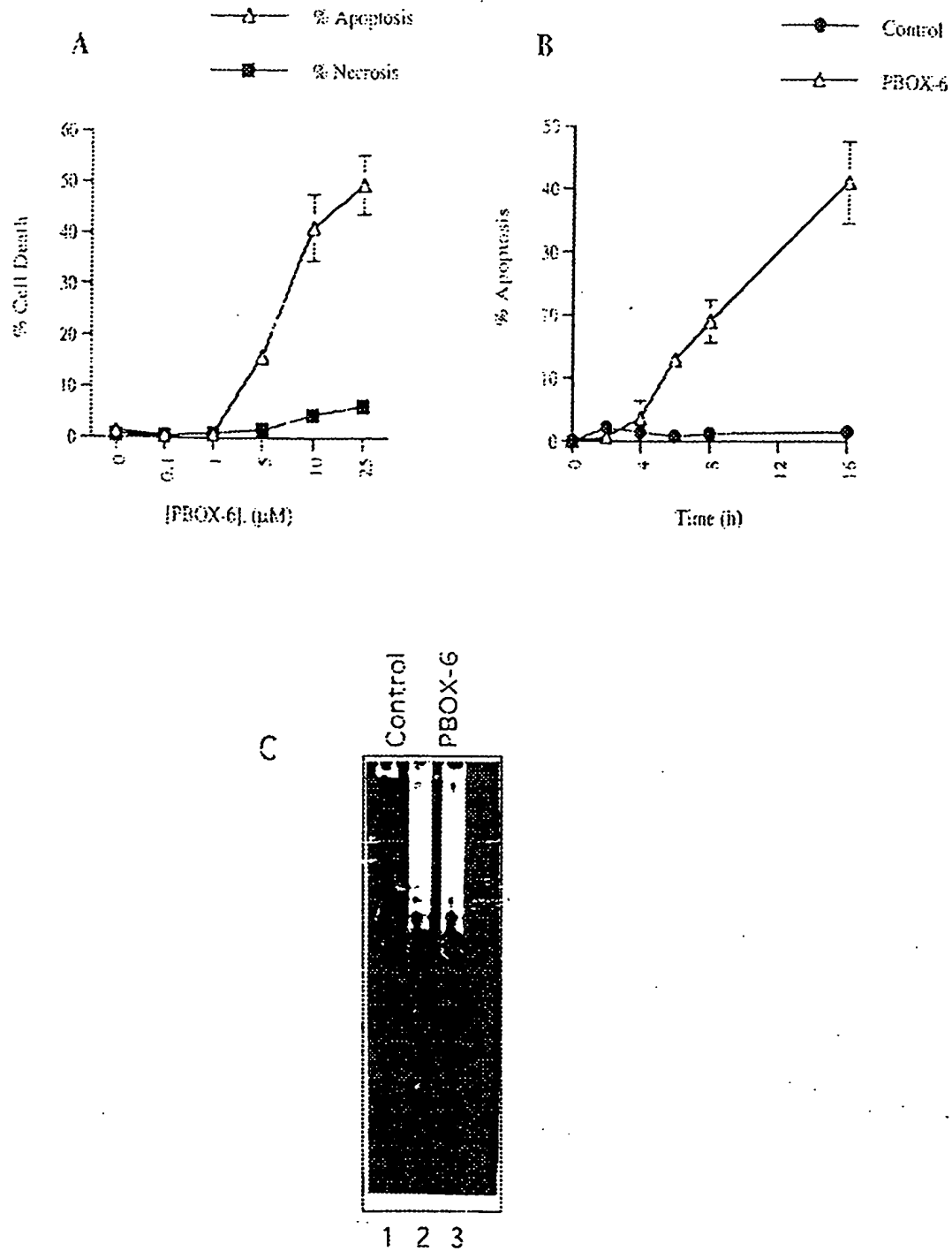


Fig. 3. PBOX-6-induced apoptosis in HL-60 cells is dose- and time-dependent and results in DNA fragmentation.

HL-60 cells were seeded at a density of 3×10^5 cells/ml and were treated with either (A) a range (0-50 μM) of concentrations of PBOX-6 for 16 hours or (B) one concentration of PBOX-6 (10 μM) for a period of 2, 4, 6, 8 and 16 hours. The percent apoptosis and necrosis was determined by cytopinning and staining the cells using the RapiDiff kit as described in the Methods section. Values represent the mean \pm SEM for three separate experiments. (C) DNA isolated from HL-60 cells, treated for 24 hours either with control (0.5% (v/v) ethanol) or PBOX-6 (10 μM) in duplicate, was analysed by gel electrophoresis.

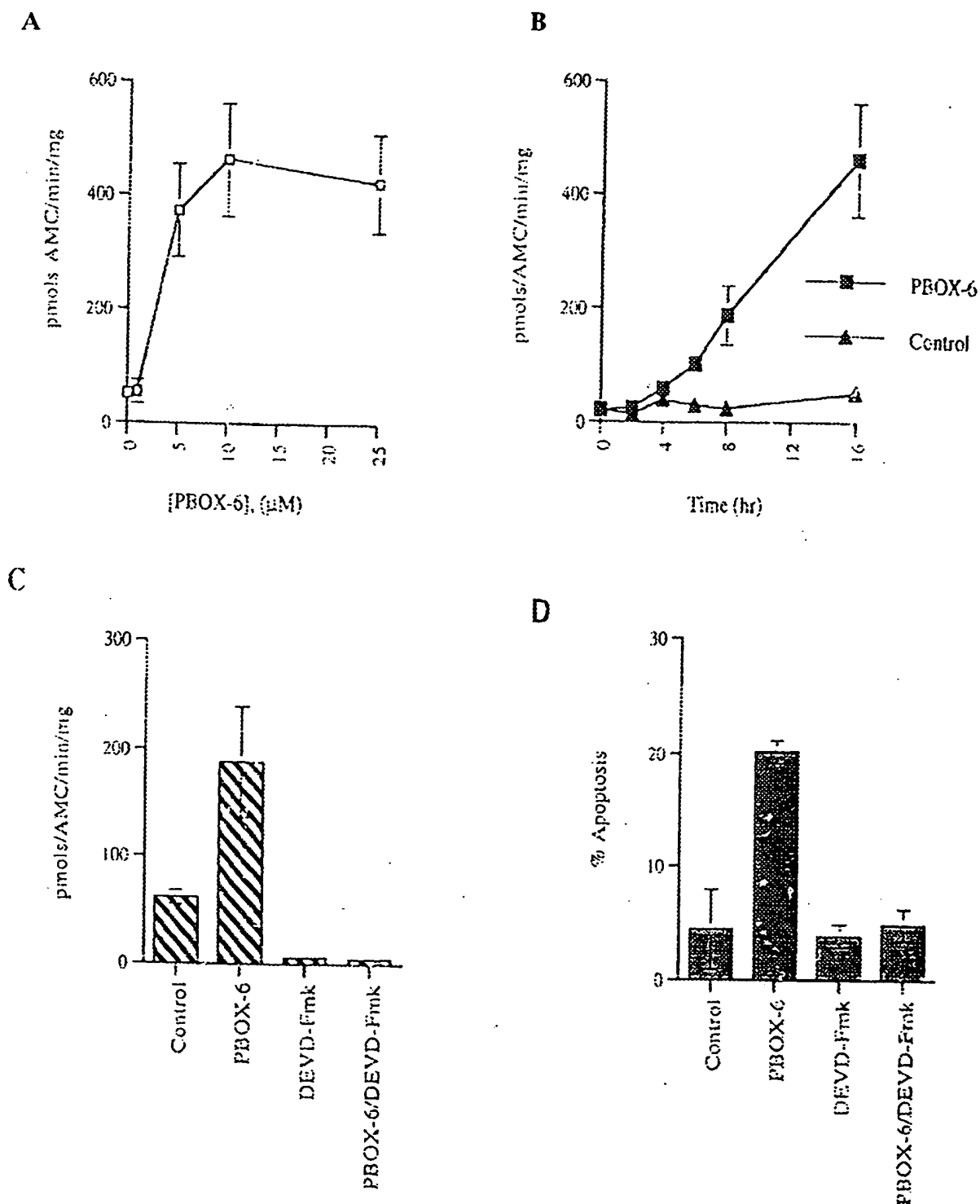


Fig. 4. PBOX-6 induces apoptosis through activation of caspase-3-like proteases. HL-60 cells were seeded at a density of 3×10^5 cells/ml and were treated with either (A) a range (0-50 μ M) of concentrations of PBOX-6 for 16 hours or (B) one concentration of PBOX-6 (10 μ M) for a period of 2, 4, 6, 8 and 16 hours or (C and D) pretreated with z-DEVD-fmk (200 μ M) for 1 h followed by treatment with PBOX-6 for a further 8h. Cytosolic extracts were prepared and assayed for caspase-3-like protease activity as described in the Methods section. The percent apoptosis and necrosis was determined by cytopinning and staining the cells using the RapiDiff kit. Values represent the mean \pm SEM of three separate experiments.

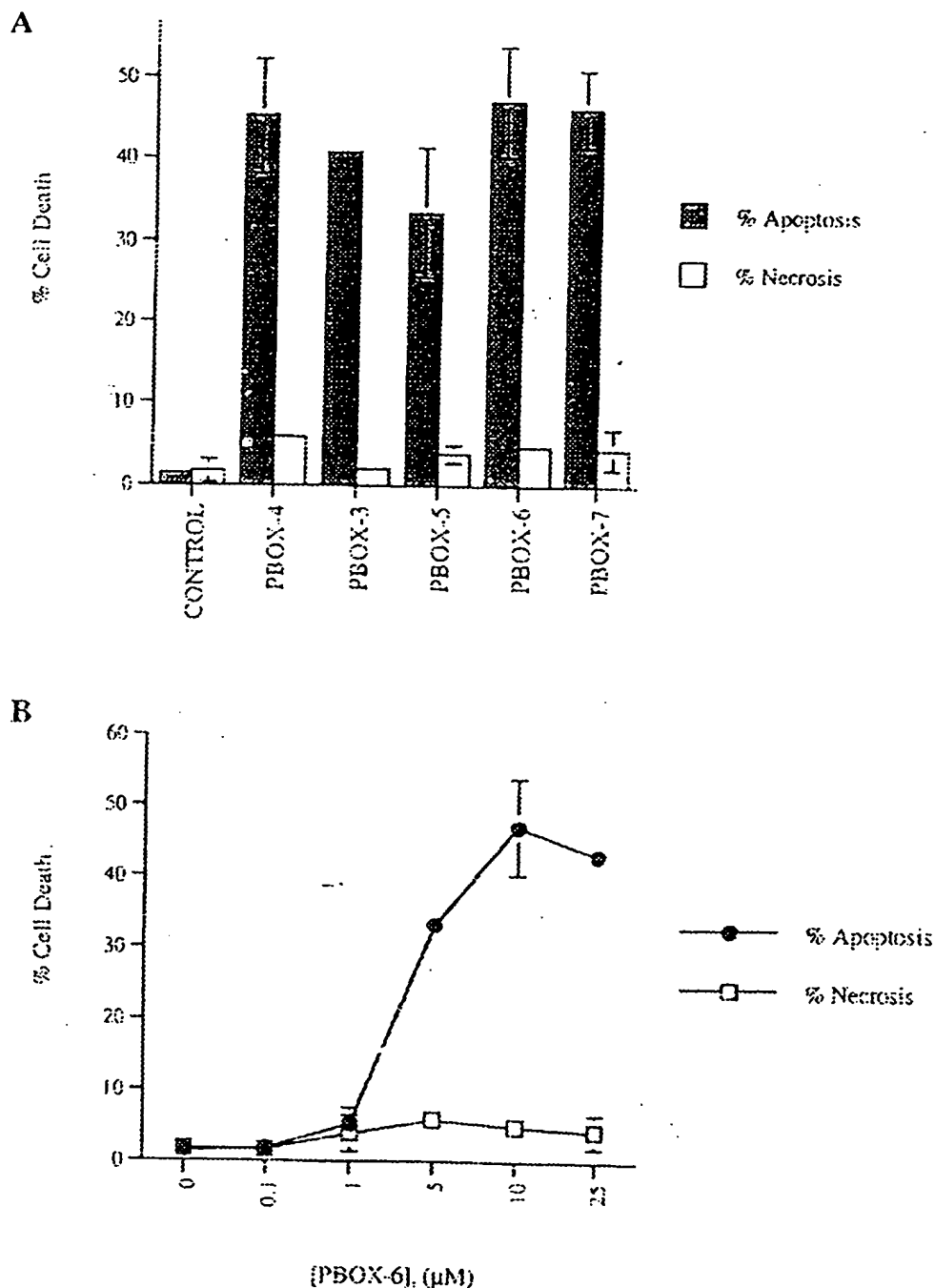


Fig. 8. Pyrrolo-1,5-benzoxazepines induce apoptosis in Jurkat cells.

Jurkat cells were seeded at a density of 3×10^5 cells/ml and were incubated with (A) either one of the indicated PBOX drugs, each at a final concentration of $10 \mu\text{M}$ or (B) a range of concentrations of PBOX-6. The control wells in each case contained 0.5% (v/v) ethanol. After 16h the percent apoptosis and necrosis was determined by cytospinning and staining the cells using the RapiDiff kit as described in the Methods section. Values represent the mean \pm SEM for three separate experiments.

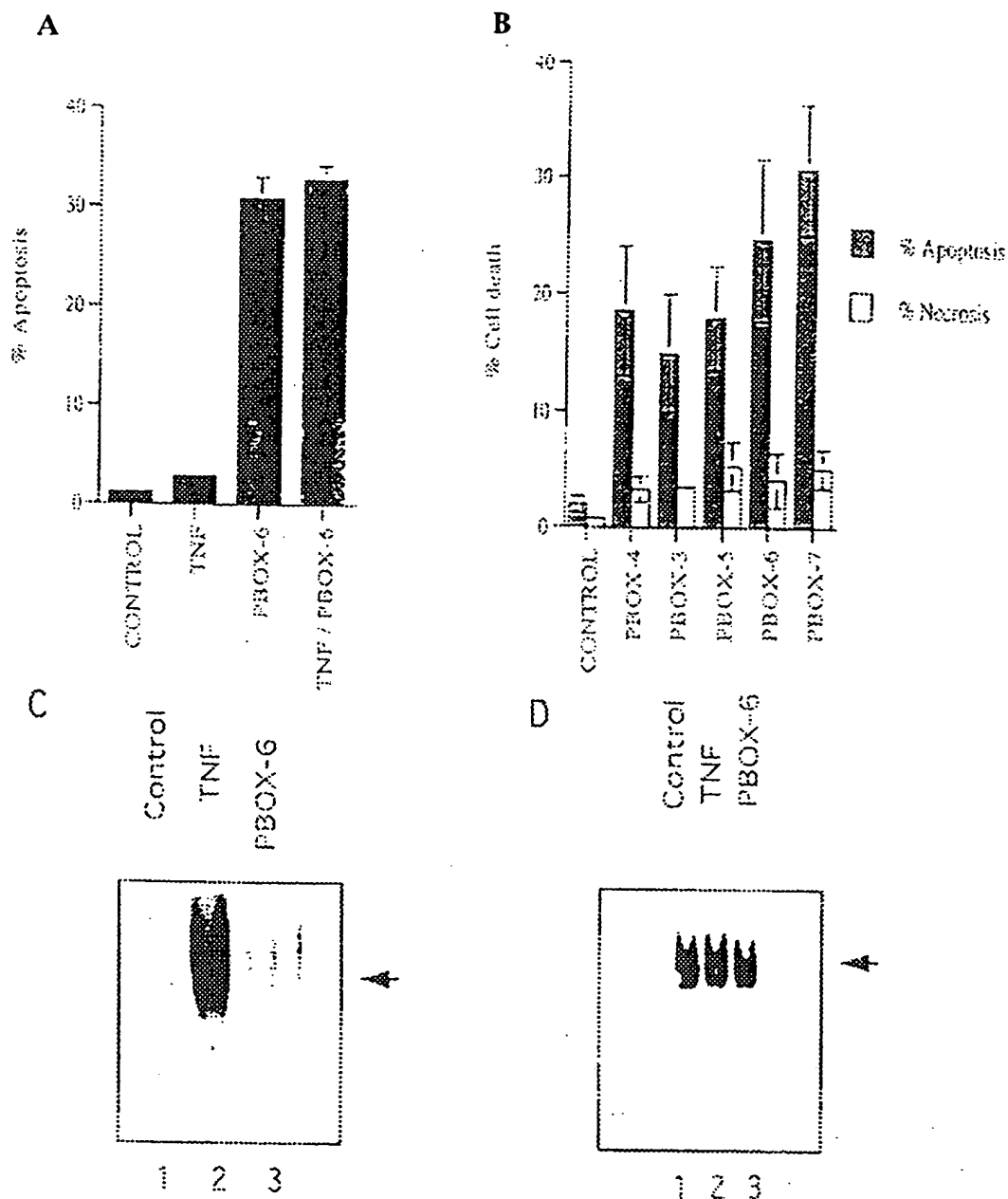


Fig. 9. Lack of involvement of NFκB in pyrrolbenzoxazepine-induced apoptosis. (A) HL-60 cells were seeded at a density of 3×10^5 cells/ml, and were pre-treated with TNFα (10ng/ml) for 1h followed by treatment with PBOX-6 for a further 16h. The control wells in each case contained 0.5% (v/v) ethanol. Values represent the mean \pm SEM of three separate experiments. (B) Same as in (A) but with Hut-78 cells incubated with either one of the indicated PBOX drugs, each at a final concentration of 10μM. Nuclear extracts (2μg) were prepared from (C) HL-60 cells treated either with control (0.5% (v/v) ethanol), TNFα (10ng/ml) or PBOX-6 (10μM) for 16 hours or (D) Same as in (C) but with Hut-78 cells. NFκB activity was then measured by EMSA described in the Methods section. The arrowhead represents NFκB-DNA. Results are representative of at least two separate experiments.

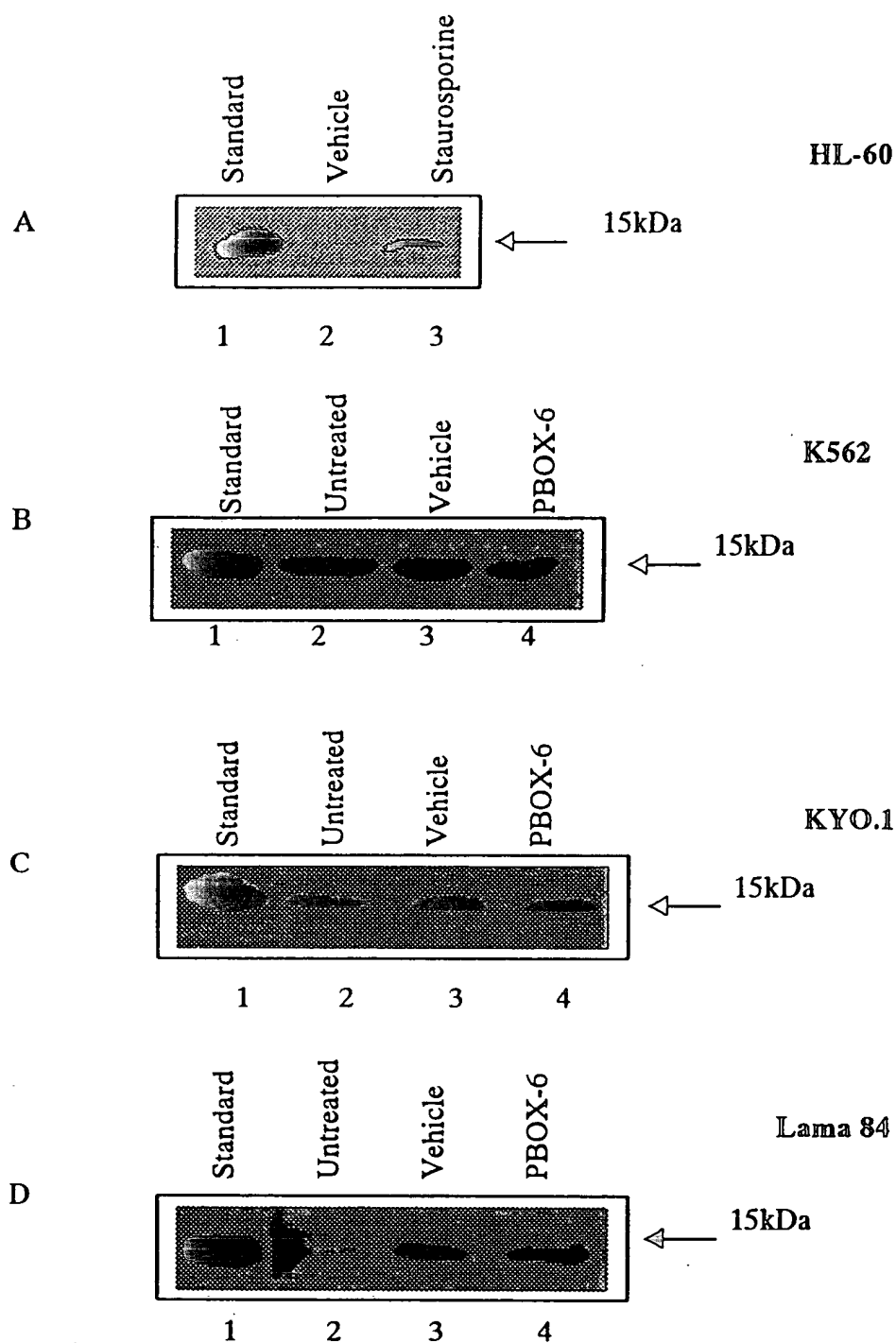


Fig. 17 Cytochrome C western blotting

Cytosolic extracts were prepared from HL-60 cells (A) which were treated with either (lane 2-3), vehicle (0.1% DMSO) or staurosporine (1 μ M) for 6 hours. K562 (B), KYO.1 (C) and Lama 84 cells (D) were treated with either (lane 2-4) control (untreated), vehicle (1% ethanol) or PBOX-6 (10 μ M) for 16 hours. Protein (30 μ g) was resolved by SDS-PAGE and probed for cytochrome C. Horse Cytochrome C was used as a standard in each case (lane 1). Results are representative of at least 2 separate experiments.

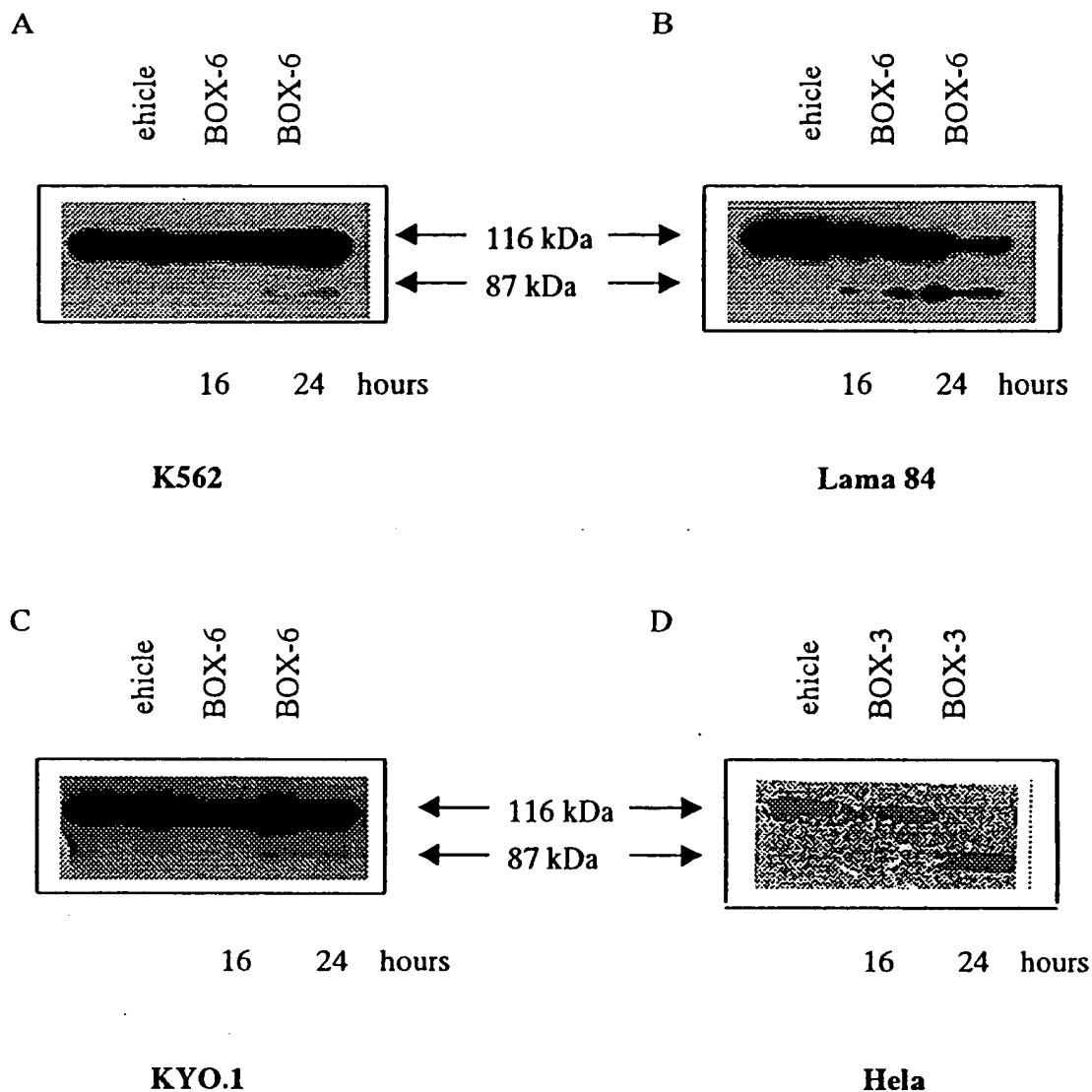


Fig. 21 Induction of PARP cleavage in CML and Hela cells following treatment with PBOX-6 and PBOX-3

Whole cell extracts from K562 (A), Lama 84 (B), and KYO.1 (C) and Hela cells (D) were prepared following treatment with either PBOX-6 (10 μ M) for 16 and 24 hours (A, B, and C) or PBOX-3 (10 μ M) for 48 hours (D). In each case a vehicle treated control was set up containing 1% ethanol. Samples were resolved by SDS-PAGE and probed with anti-PARP antibody. Results are representative of at least 2 experiments.

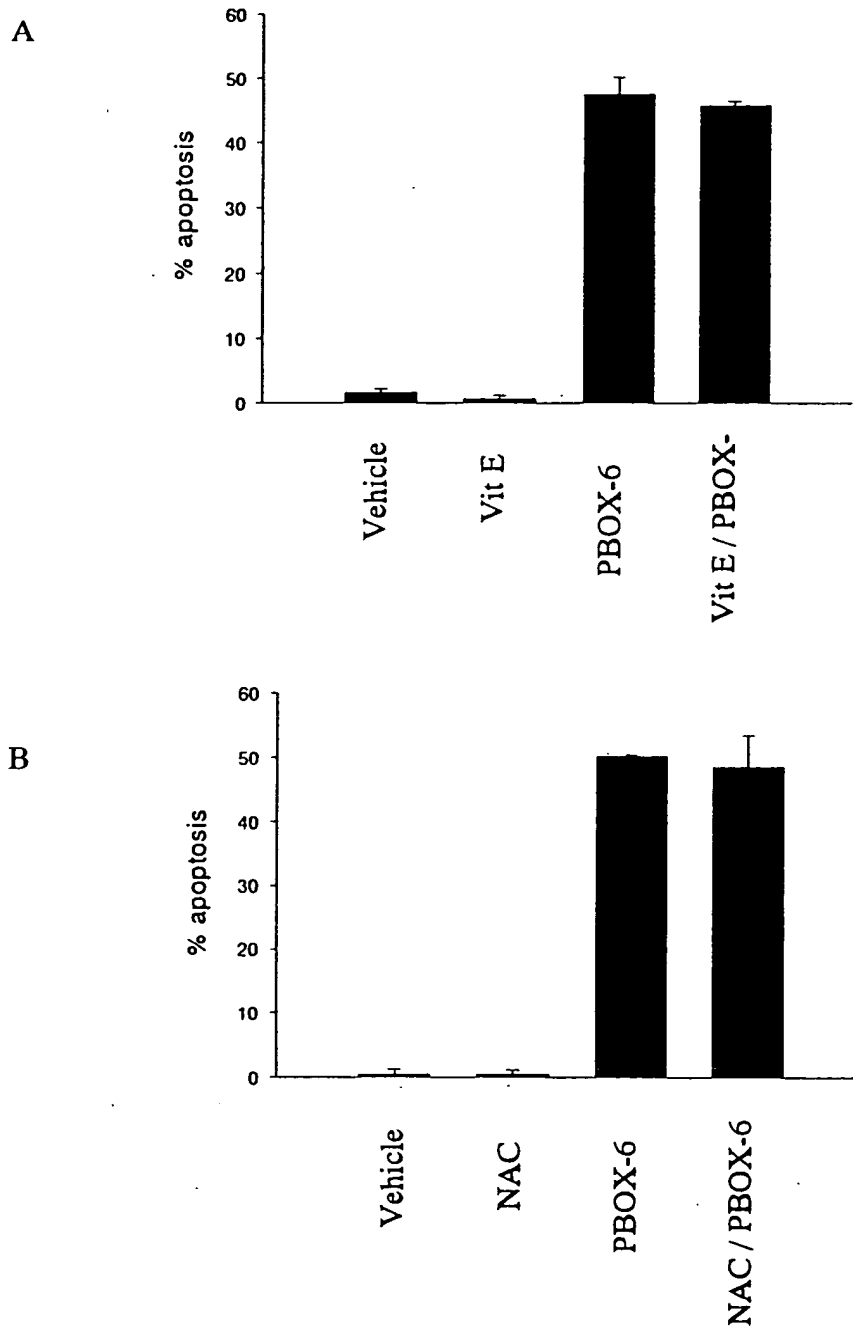


Fig. 22 Antioxidants fail to protect against PBOX-6-induced apoptosis in K562 cells. K562 cells were seeded at 3×10^5 cells per ml and treated with (A) either vehicle (1% PBS, 0.1% ethanol), Vitamin E (100 μ M) for 40 hours, PBOX-6 (10 μ M) for 16 hours or a pretreatment of Vitamin E for 24 hours followed by PBOX-6 for a further 16 hours. In (B) cells were treated with either vehicle (25mM Tris, 0.1% ethanol), N-Acetylcysteine (NAC) (5mM) for 17 hour, or a pretreatment of NAC for 1 hour followed by PBOX-6 for a further 16 hours. Percent apoptosis was determined by RapiDiff staining. Results represent the mean \pm SEM of 3 separate experiments.

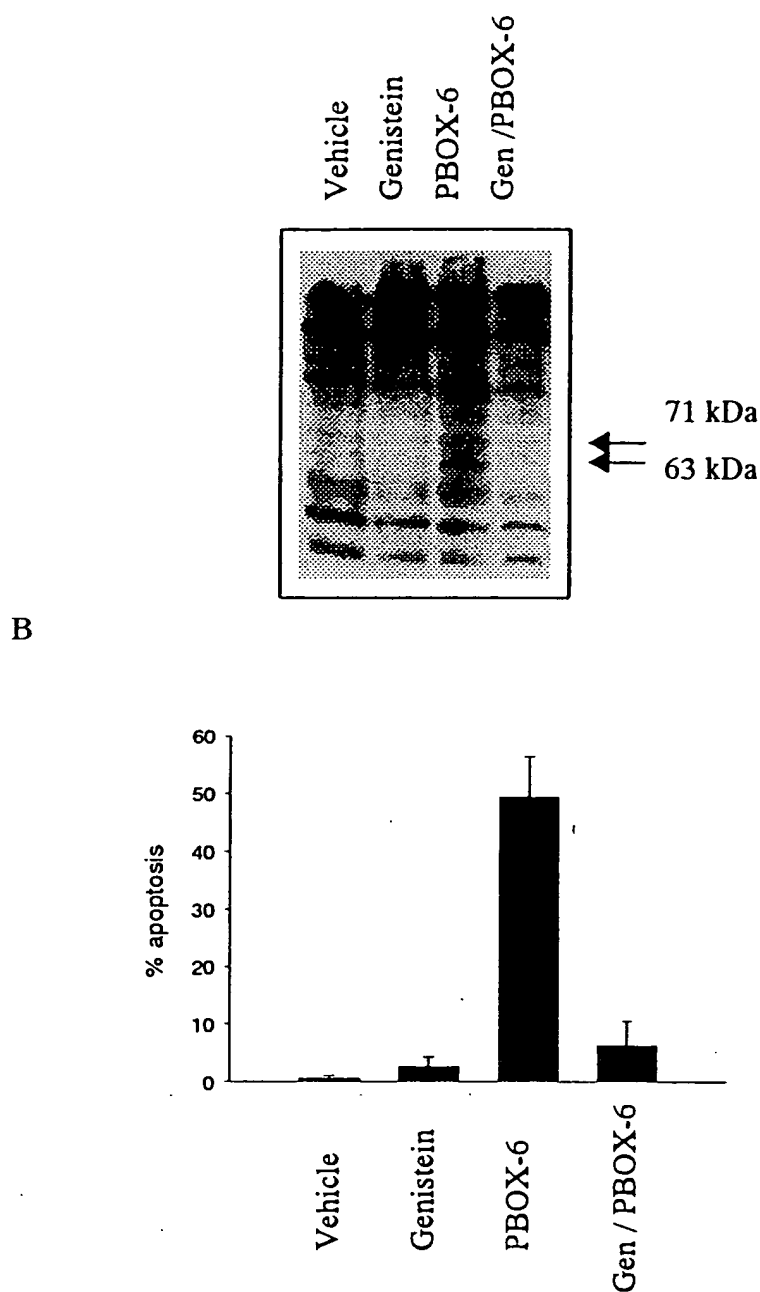


Fig. 24 Pretreatment of K562 cells with the tyrosine kinase inhibitor, Genistein, prevents protein tyrosine phosphorylation and inhibits apoptosis induced by PBOX-6. K562 cells were seeded at (A) 5×10^6 cells per sample or (B) 3×10^5 cells per ml and pretreated with genistein ($100 \mu\text{M}$) for 1 hour prior to treatment with PBOX-6 ($10 \mu\text{M}$) for a further 16 hours. In (A) cytosolic extracts were prepared as described in Section 2.10. Protein ($40 \mu\text{g}$) was resolved by SDS-PAGE and probed with anti-phosphotyrosine antibody. Results are representative of at least 3 experiments. In (B) percent apoptosis was determined by RapiDiff staining. Results represent the mean \pm SEM of 3 separate experiments.

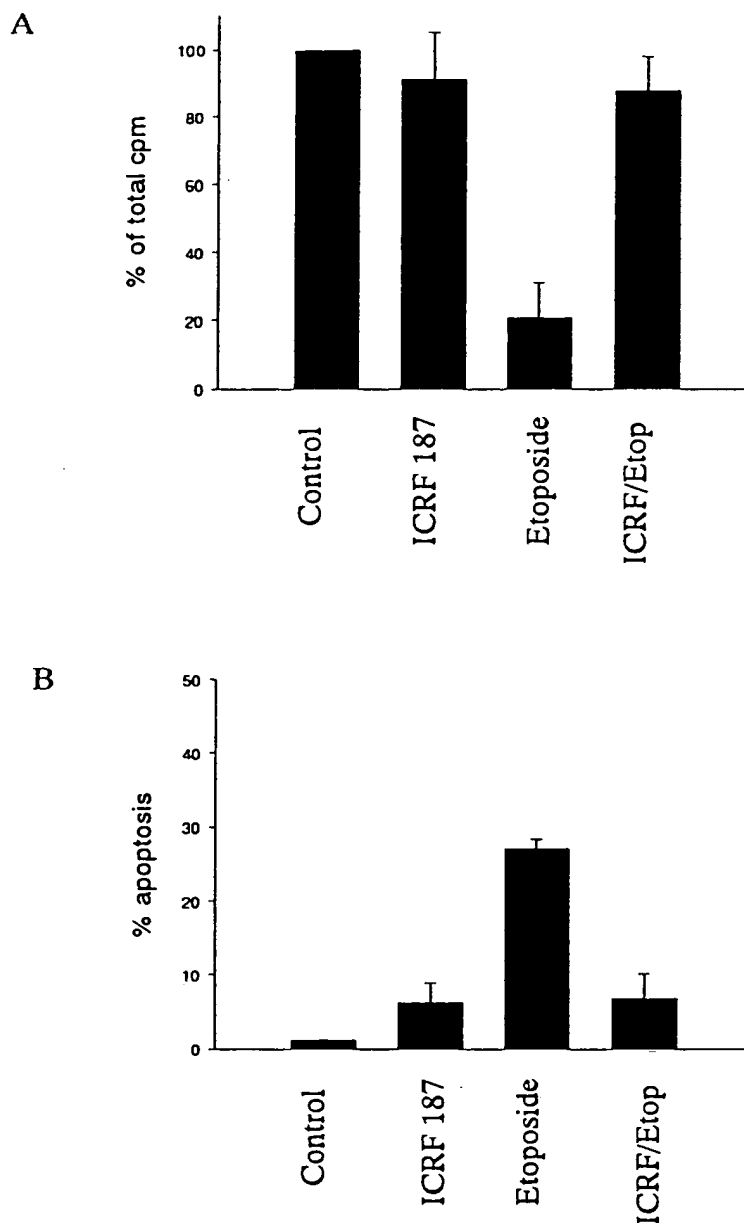


Fig. 28 Pretreatment of Jurkat cells with ICRF 187 inhibits etoposide induced DNA strand breaks and protects against apoptosis.

Jurkat cells were either set up as outlined in Section 1.11 (A) or seeded at 3×10^5 cells per ml (B) and treated with (A) control (0.1% ethanol:DMSO (1:1)), ICRF 187 (200 μ M) for 1 hour, etoposide (2.5 μ M) for 1 hour or a pretreatment of ICRF 187 for 1 hour prior to treatment with etoposide for a further hour. Cells were lysed onto filters and eluted overnight. In (B) cells were treated with either vehicle (0.1% ethanol:DMSO (1:1)), ICRF 187 (200 μ M) for 17 hours, etoposide (2.5 μ M) for 16 hours or a pretreatment of ICRF 187 for 1 hour followed by etoposide for a further 16 hours. Percent apoptosis was determined by RapiDiff staining. Values represent the mean \pm range of 2 separate experiments.

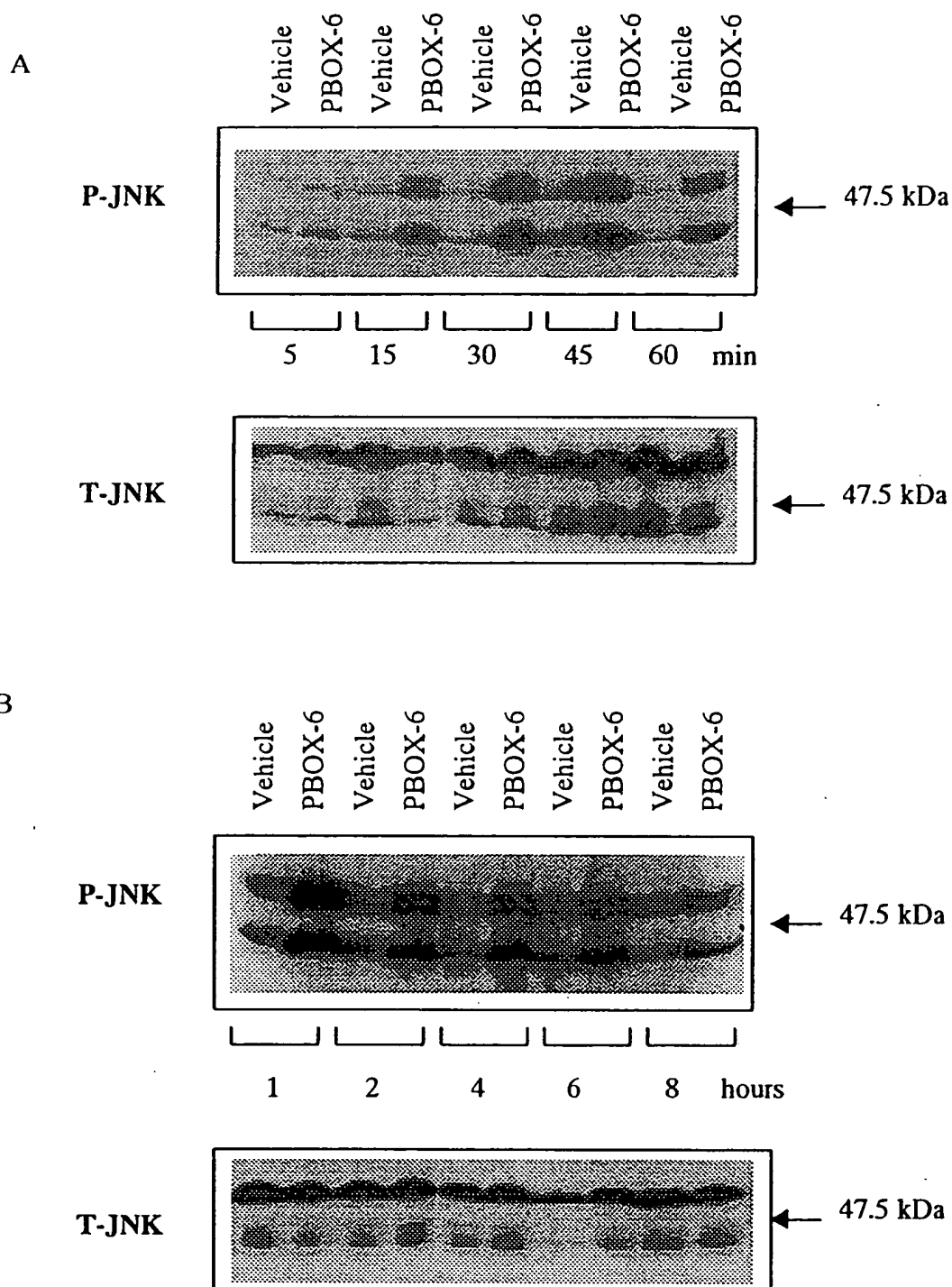


Fig. 31 PBOX-6 induces transient activation of JNK in K562 cells.

K562 cells were seeded at 6×10^6 cells per sample and treated with either vehicle (1% ethanol) or PBOX-6 (10 μ M) for (A) 5, 15, 30, 45 and 60 min, or (B) 1, 2, 4, 6 and 8 hours. Whole cell extracts were prepared and protein (40 μ g) was resolved by SDS-PAGE. Blots were probed with anti-JNK phospho antibody and were then stripped and re-probed with anti-JNK total as a loading control. Results are representative of two separate experiments.

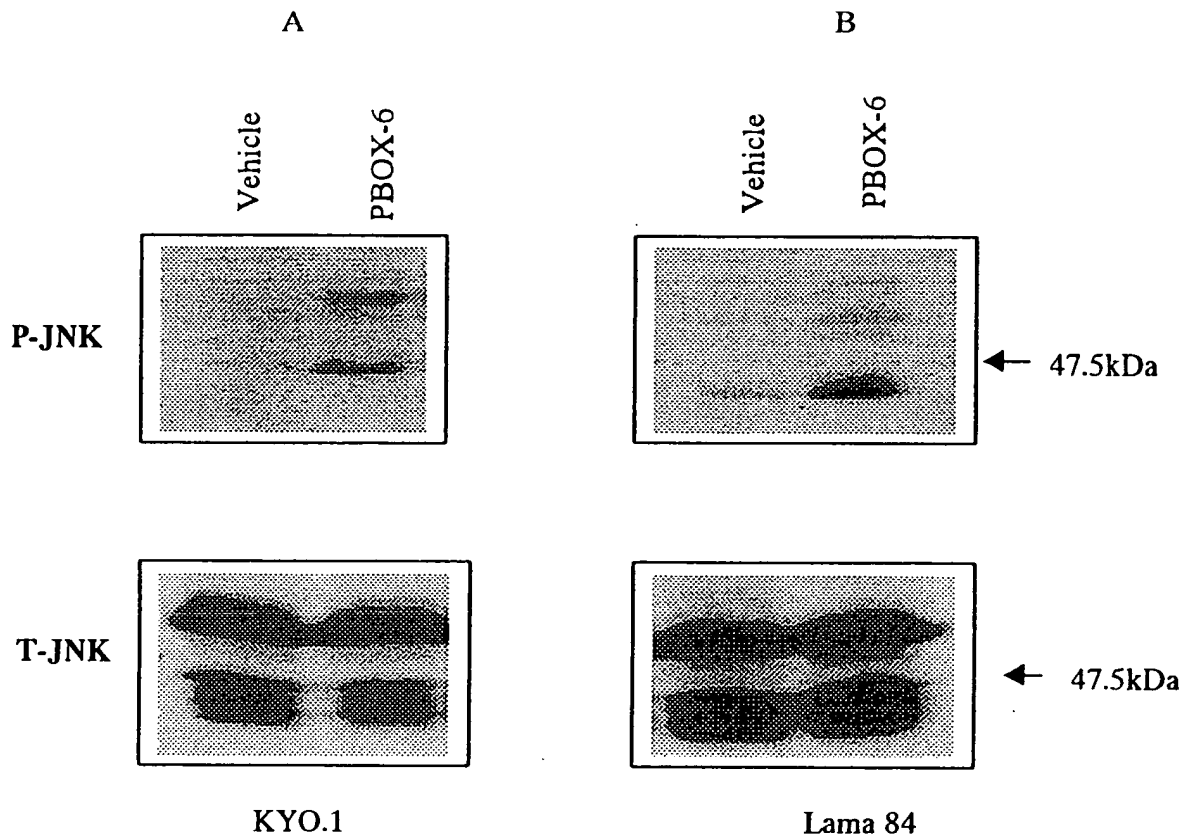


Fig. 32 PBOX-6 induces activation of JNK in KYO.1 and Lama 84 cells.

KYO.1 (A) and Lama 84 cells (B) were seeded at 6×10^6 cells per sample and treated with either vehicle (1% ethanol) or PBOX-6 (10 μ M) for 45 minutes. Whole cell extracts were prepared and protein (50 μ g) was resolved by SDS-PAGE. Blots were incubated with anti-JNK phospho antibody and then stripped and re-probed with anti-JNK total antibody as a loading control. Results are representative of two separate

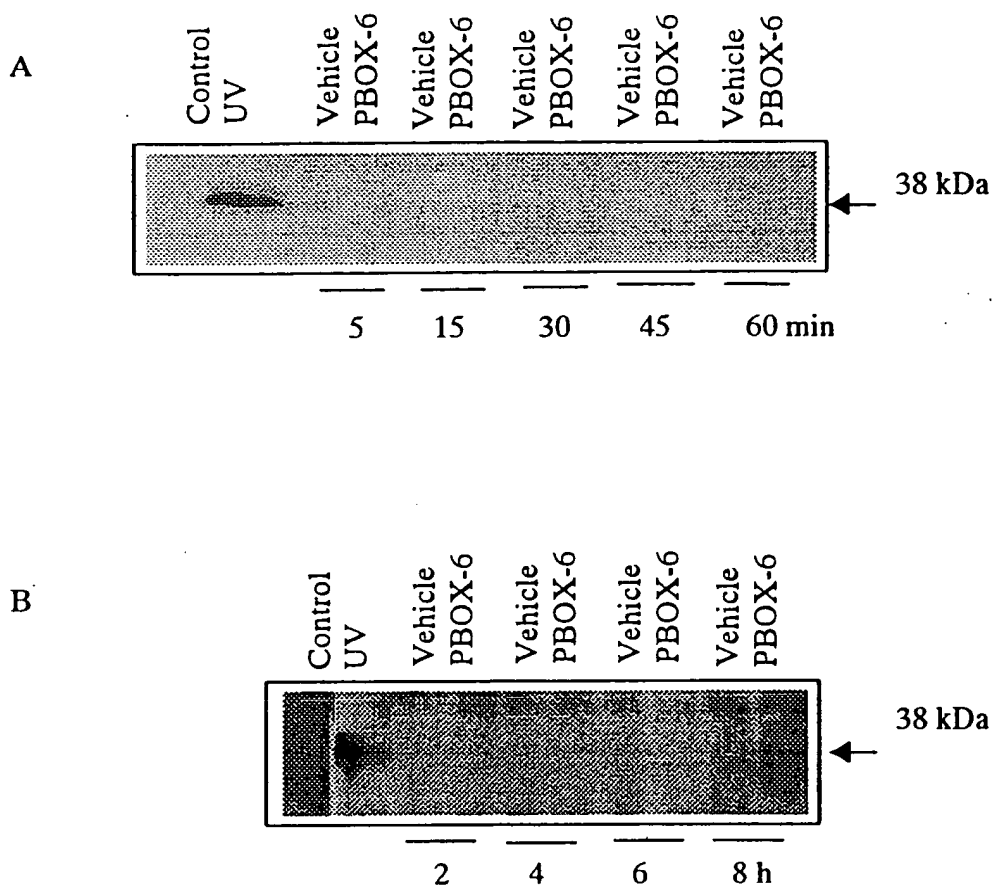


Fig. 33 Lack of activation of p38 in K562 cells in response to PBOX-6.

Cells were seeded at 6×10^6 cells/flask and Jurkat cells (lanes 1-2) were UV irradiated for 2 min and incubated at 37°C for a further 2h. K562 cells (lanes 3-12) were treated with vehicle (1% (v/v) ethanol) or PBOX-6 ($10\mu\text{M}$) for either (A) 5, 15, 30, 45 and 60 min or (B) 2, 4, 6 and 8h. Whole cell extracts were prepared and equal amounts of protein were resolved by SDS-PAGE and probed with a phospho-specific p38 antibody. Results are representative of two separate experiments.

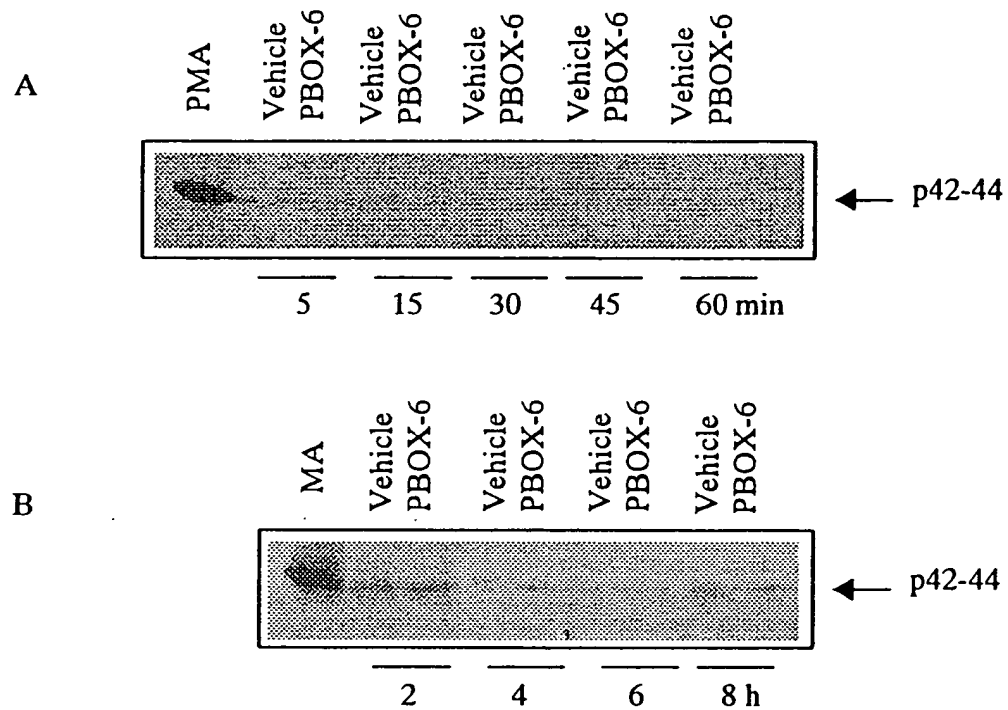


Fig. 34 Lack of activation of p42-44 in K562 cells in response to PBOX-6

K562 cells were seeded at 5×10^6 cells/flask and treated with PMA (100nM) for 30 min (lane 1) as a positive control or vehicle (1% (v/v) ethanol) or PBOX-6 (10 μ M) for (A) 5, 15, 30, 45 and 60 min (lanes 2-11) or (B) 2, 4, 6 and 8h (lanes 2-11). Whole cell extracts were prepared and equal amounts of protein (50 μ g) was resolved by SDS-PAGE and probed with a phospho-specific p42-44 antibody. Results are representative of two separate experiments.

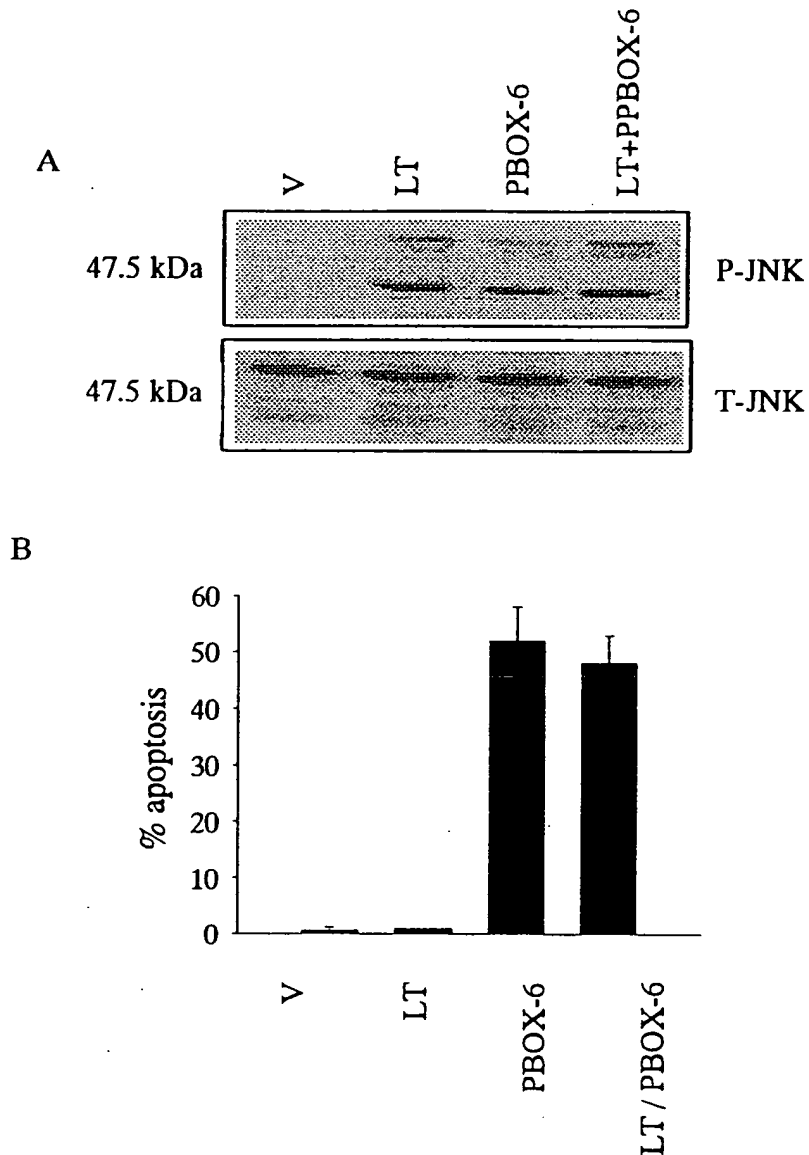


Fig. 35 Pretreatment of K562 cells with an inhibitor of Rac 1, Lethal toxin, failed to protect against PBOX-6 induced JNK activation and apoptosis.

K562 cells were seeded at either (A) 6×10^6 cells per sample or (B) 3×10^5 cells per m and pretreated with either (A) lethal toxin (500ng/ml) for 3 hours followed by PBOX-6 (10 μ M) for a further 45 mins. Protein (50 μ g) was resolved by SDS-PAGE and probe with anti-JNK-phospho antibody. Blots were stripped and re-probed with anti-JNK tota antibody as a loading control. In (B) cells were pretreated with lethal toxin (500ng/ml for 1 hour prior to treatment with PBOX-6 (10 μ M) for a further 16 hours. Cells were spun onto a slide and percent apoptosis was determined using RapiDiff staining. Results are representative of 2 separate experiments.

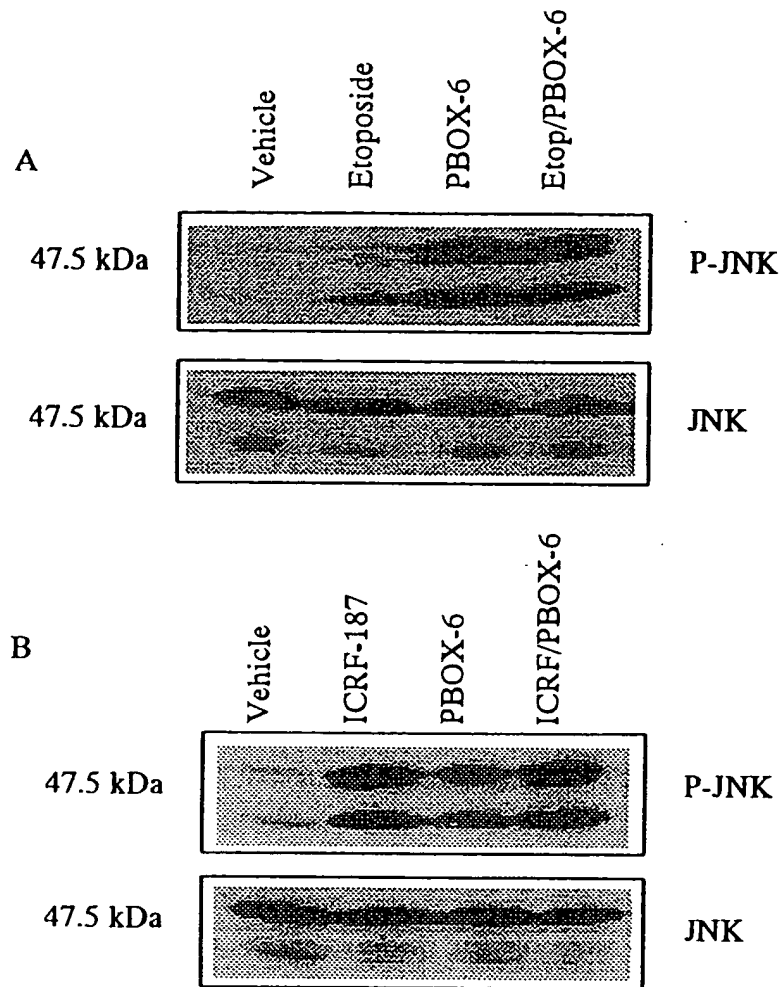


Fig. 36. Activation of JNK lies upstream of a requirement for Topo II in the pathway by which PBOX-6 induces apoptosis in K562 cells.

K562 cells were seeded at 6×10^6 cells per sample and pretreated with either (A) etoposide ($50 \mu\text{M}$) or (B) ICRF 187 ($200 \mu\text{M}$) for 1 hour prior to treatment with PBOX-6 ($10 \mu\text{M}$) for a further 45 min. Whole cell extracts were prepared and protein ($50 \mu\text{g}$) was resolved by SDS-PAGE. Blots were probed with anti-JNK phospho antibody, stripped and re-probed with anti-JNK total antibody as a loading control. Results are representative of at least two separate experiments.

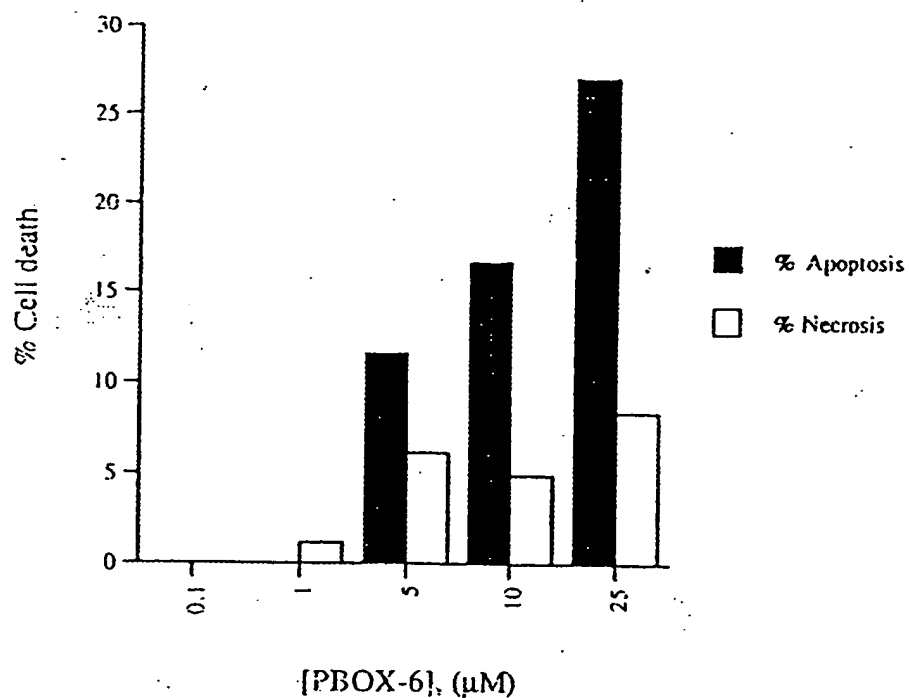


Fig. 37 PBOX-6 induces apoptosis in MCF-7 cells.

MCF-7 cells were seeded at a density of 6×10^6 cells/ml and were incubated with a range of concentrations of PBOX-6. The control wells in each case contained 0.5% (v/v) ethanol. After 24h the percent apoptosis was determined by cytopinning the cells onto a glass slide and staining them using the RapiDiff kit as described in the Methods section